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Filter Extraction and Ames Bioassay Results for EPA (Environmental Protection Agency) Particulate Samples

Southwest Research Inst., San Antonio, TX

Prepared for

Environmental Protection Agency, Ann Arbor, MI

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Filter Extraction and Ames Bioassay Results for EPA Particulate Samples

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Emission Control Technology Division
2565 Plymouth Road
Ann Arbor, Michigan 48105

August 1984

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FOREWORD

This project was conducted for the U.S. Environmental Protection Agency by the Department of Emissions Research, Southwest Research Institute. The work was carried out between September 1983 and May 1984 under EPA Contract No. 68-03-3162, Work Assignment Number 2. It was identified within Southwest Research Institute as Project 03-7338-002. The EPA Project Officers were Mr. Robert J. Garbe and Mr. Craig A. Harvey, and the Branch Technical Representative was Mr. Craig A. Harvey, both of the Characterization and Technical Applications Branch, Emission Control Technology Division, Environmental Protection Agency, 2565 Plymouth Road, Ann Arbor, Michigan. The Southwest Research Institute Project Manager was Charles T. Hare, and the Project Leader and Principal Investigator was Mary Ann Warner-Selph.

ABSTRACT

This report describes filter extractions and Ames bioassay of filter extracts performed for the Emission Control Technology Division of the Environmental Protection Agency. Eight sets of particulate-loaded filters were provided to SwRI by the sponsor. The filters were soxhlet-extracted in methylene chloride, and the extracts were dried and weighed. The organic extracts were analyzed using the Ames bioassay at Southwest Foundation for Biomedical Research (SFBR), formerly Southwest Foundation for Research and Education. The data were analyzed using linear and non-linear regression methods.

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I. SUMMARY

Results from the Ames bioassay are reported in this section. The data that were analyzed using linear regression are reported in Table 1. Samples TAEB-82-0191 and TAEB-83-0020 caused the greatest Ames response of the eight samples. TAEB-82-0191, produced by a VW during the LA-4 cycle with a JM-13 trap, was most mutagenic in TA 1537, TA 1538, and TA 98. Sample TAEB-83-0020, produced by a Mercedes equipped with an NGK-2 trap and operated over the LA-4, was most mutagenic in TA 98 and TA 100. The greatest Ames response was observed in TA 104 for samples analyzed in the five additional tester strains (TA 97, TA 102, TA 104, TA 98NR, and TA 98/1,8-DNP_6).

Ames data analyzed with the non-linear model are summarized in Table 2. The highest Ames response was observed with samples TAEB-82-0191 and TAEB-84-0020, similar to results calculated by linear regression. Sample -0191 produced the highest revertants per microgram of extract in tester strains TA 1537, TA 1538, and TA 98, and sample -0020 had the highest response in TA 1538, TA 98, and TA 100. TAEB-82-0221 and TAEB-82-0161 produced consistently low Ames results in most of the strains. The Ames response of the samples tested in the five extra tester strains did not show marked differences between strains. The average response in TA 104 was somewhat higher, however, than responses in TA 97, TA 102, TA 98NR, and TA 98/1,8-DNP_6.

Ames dose-response data calculated by the non-linear model were generally higher than those determined by linear regression. As discussed above, however, the same trends occurred regardless of the method chosen for data analysis.

TABLE 1. SUMMARY OF AMES BIOASSAY OF EXTRACTS, DOSE-RESPONSE RELATIONSHIP CALCULATED BY LINEAR REGRESSION

	rev/µg																			
	TA 1535		TA 1537		TA 1538		TA 98		TA 100		TA 97[a]		TA 102[a]		TA 104[a]		TA 98NR[a]		TA 98/1,8-DNP6[a]	
Sample Identification	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
TAEB-81-0781 VW LA-4 baseline	-0.02	0.06	0.67	1.12	2.30	1.96	3.32	2.01	3.07	1.82	1.52	0.74	1.13	1.55	7.32	2.51	0.88	1.01	1.99	0.66
TAEB-82-0191[b] VW LA-4 JM-13 trap	-0.05	1.65	5.53	5.16	15.53	9.73	9.56	7.53	4.57	2.98										
TAEB-82-0101[b] VW LA-4 JM-13 trap	-0.11	0.40	1.82	1.87	5.29	4.55	3.90	2.75	3.19	2.39										
TAEB-82-0131[b] VW Regen JM-13 trap	--[c]	--[c]	--[c]	--[c]	--[c]	--[c]	2.00	7.64	1.52	2.99										
TAEB-82-0221 Toyota LA-4 BS2-1 trap	0.00	0.06	0.27	0.32	0.58	0.74	0.72	1.14	0.89	0.85	0.43	0.54	0.50	0.55	1.30	1.05	0.30	0.56	0.41	0.19
TAEB-82-0161 Toyota LA-4 BS2-1 trap	-0.01	0.04	0.20	0.28	0.26	0.68	0.74	0.72	0.96	0.54	0.33	0.87	0.58	0.33	1.62	1.27	0.26	0.56	0.38	0.09
TAEB-84-0010 Mercedes LA-4 baseline	0.04	0.26	0.54	0.60	1.18	1.12	1.70	1.32	2.84	3.44	0.46	0.67	1.04	0.83	2.61	1.32	0.81	1.00	1.35	0.19
TAEB-84-0020 Mercedes LA-4 NGK-2 trap	-0.03	0.36	4.98	2.10	3.52	3.17	12.22	3.20	14.06	12.15										

[a] No repeat analysis
[b] Insufficient sample to perform repeat analysis
[c] Insufficient sample to perform analysis in TA 1535, TA 1537 and TA 98

TABLE 2. SUMMARY OF AMES BIOASSAY OF EXTRACTS, DOSE-RESPONSE RELATIONSHIP CALCULATED BY EPA-RTP NON-LINEAR MODEL

	rev/µg																			
Sample Identification	TA 1535		TA 1537		TA 1538		TA 98		TA 100		TA 97[a]		TA 102[a]		TA 104[a]		TA 98NR[a]		TA 98/1,8-DNP$_6$[a]	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
TAEB-81-0781 VW LA-4 baseline	0.04	0.47	3.11	3.57	11.0	4.97	12.8	5.03	7.48	3.57	5.00	0.75	2.86	2.34	17.2	6.77	2.63	3.71	8.96	0.89
TAEB-82-0191[b] VW LA-4 JM-13 trap	1.43	4.93	15.8	43.6	143	37.7	28.5	19.4	22.5	8.92										
TAEB-82-0101[b] VW LA-4 JM-13 trap	0.76	2.81	8.24	15.9	27.9	36.0	14.9	7.66	13.9	6.64										
TAEB-82-0131[b] VW Regen JM-13 trap	--[c]	--[c]	--[c]	--[c]	--[c]	--[c]	4.54	24.7	4.47	9.36										
TAEB-82-0221 Toyota LA-4 BS2-1 trap	0.00	0.36	1.13	0.32	1.65	0.86	2.26	1.26	1.19	1.03	1.09	1.06	2.54	1.82	3.41	2.48	0.30	5.28	0.95	0.46
TAEB-82-0161 Toyota LA-4 BS2-1 trap	0.05	0.04	0.44	0.35	0.52	1.05	1.69	1.33	1.58	1.19	0.41	0.88	0.98	0.34	3.45	1.38	0.26	0.77	0.56	0.13
TAEB-84-0010 Mercedes LA-4 baseline	0.00	0.46	7.32	1.01	5.50	1.99	5.82	2.74	7.96	7.58	2.18	1.93	20.6	1.34	7.19	2.60	1.26	2.92	9.25	0.16
TAEB-84-0020 Mercedes LA-4 NGK-2 trap	0.00	0.86	2.48	7.36	4.17	28.8	33.5	8.39	60.3	3.21										

[a] No repeat analysis
[b] Insufficient sample to perform repeat analysis
[c] Insufficient sample to perform analysis in TA 1535, TA 1537 and TA 98

II. INTRODUCTION

This project provided for the extraction of 23 filters to produce eight samples for Ames bioassay analysis. The filters were generated by EPA and shipped to SwRI for methylene chloride extraction. A list of all filters extracted by SwRI is shown in Table 3. Extraction results, in mass and percent of particulate mass, are also listed in the Table.

The program initially called for a full Ames analysis of the methylene chloride extractables. This plan included analysis in five tester strains in triplicate, with and without metabolic activation, with a repeat. The test procedure required a minimum of 145 mg of extract. Four of the samples contained insufficient extract to perform the full bioassay. Samples -0191 and -0101 contained only enough extract to perform initial analyses with no repeat. Sample -0131 was tested in only 2 tester strains, TA 98 and TA 100, without a repeat, and at reduced dosage levels. Sample -0020 was tested at reduced dosage, but a repeat analysis was included.

With the approval of Craig Harvey, EPA Branch Technical Representative, additional analyses of the four largest samples (having more than minimum required mass) were performed. These tests were performed to bring the level of effort up to that in the original test plan. Samples -0781, -0221, -0161, and -0010 were analyzed in five additional tester strains without a repeat: TA 97, TA 102, TA 104, TA 98NR, and TA 98/1,8-DNP_6. TA 97 is responsive to aridine compounds, and is recommended as a replacement for TA 1537.[1] Tester strains TA 102 and TA 104 are especially sensitive to nitro-PAH compounds. TA 98NR is insensitive to 1-nitro-PAH compounds, but sensitive to dinitro compounds. The reverse response pattern occurs with tester strain TA 98/1,8-DNP_6.

Ames results were processed by two models, linear regression and a non-linear model. SFBR calculated the slope (rev/μg) using linear regression. For samples in which the extract dose became toxic to the bacteria, the dosages were not included in the slope calculation. The non-linear model, however, includes all data points, regardless of toxicity. Ames data were processed using a non-linear computer program at EPA-RTP.

Ames results calculated by linear regression are listed by individual test in Table 4. Data calculated by the non-linear method are listed by individual tests in Table 5. As mentioned previously, summaries of results calculated by linear regression and by the non-linear method are reported in Tables 1 and 2, respectively.

TABLE 3. RESULTS OF METHYLENE CHLORIDE EXTRACTIONS ON FILTERS PROVIDED BY EPA

Filter Number	Sample Extract Number	Sample Identification	Particulate Wt. (g)	Extract Wt. (g)	Percent Extractables
TAEB-81-0780 TAEB-81-0800	TAEB-81-0781	VW LA-4 baseline	3.53	0.8793	24.9%
TAEB-82-0190 TAEB-82-0200 TAEB-82-0210	TAEB-82-0191	VW LA-4 JM-13 trap	1.41	0.0784	5.6
TAEB-82-0100 TAEB-82-0110 TAEB-82-0120	TAEB-82-0101	VW LA-4 JM-13 trap	1.35	0.0778	5.8
TAEB-82-0130 TAEB-82-0140 TAEB-82-0150	TAEB-82-0131	VW Regen. JM-13 trap	11.35	0.0188	0.2
TAEB-82-0220 TAEB-82-0230 TAEB-82-0240	TAEB-82-0221	Toyota LA-4 BS2-1 trap	1.82	0.5508	30.3
TAEB-82-0160 TAEB-82-0170 TAEB-82-0180	TAEB-82-0161	Toyota LA-4 BS2-1 trap	2.45	0.6395	26.1
83-0640 83-0650 83-0660	TAEB-84-0010	Mercedes LA-4 baseline	3.58	0.3198	8.9
810 820 830	TAEB-84-0020	Mercedes LA-4 NGK-2 trap	1.28	0.1233	9.6

TABLE 4. RESULTS OF INDIVIDUAL AMES TESTS OF EXTRACTS, DOSE-RESPONSE RELATIONSHIP CALCULATED BY LINEAR REGRESSION

Sample Identification	Test	rev/µg																			
		TA 1535		TA 1537		A 1538		TA 98		TA 100		TA 97[a]		TA 102[a]		TA 104[a]		TA 98NR[a]		TA98/1,8-DNP6[a]	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
TAEB-81-0781	initial	-0.02	0.04	1.23	1.01	3.15	2.36	3.40	2.07	3.20	2.23	1.52	0.74	1.13	1.55	7.32	2.51	0.88	1.01	1.99	0.66
VW LA-4 baseline	repeat	-0.03	0.08	0.11	1.23	1.45	1.55	3.23	1.95	2.94	1.41										
	avg.	-0.02	0.06	0.67	1.12	2.30	1.96	3.32	2.01	3.07	1.82										
TAEB-82-0191 VW LA-4 JM-13 trap	initial	-0.05	1.65	5.53	5.16	15.53	9.73	9.56	7.53	4.57	2.98										
TAEB-82-0101 VW LA-4 JM-13 trap	initial	-0.11	0.40	1.82	1.87	5.29	4.55	3.90	2.75	3.19	2.39										
TAEB-82-0131 VW Regen. JM-13 trap	initial	--[c]	--[c]	--[c]	--[c]	--[c]	--[c]	2.00	7.64	1.52	2.99										
TAEB-82-0221	initial	0.00	0.09	0.29	0.33	0.70	0.73	0.53	1.23	1.05	0.84	0.43	0.54	0.50	0.55	1.30	1.05	0.30	0.56	0.41	0.19
Toyota LA-4 BS2-1 trap	repeat	0.00	0.04	0.25	0.32	0.46	0.76	0.90	1.05	0.73	0.86										
	avg.	0.00	0.06	0.27	0.32	0.58	0.74	0.72	1.14	0.89	0.85										
TAEB-82-0161	initial	-0.02	0.04	0.18	0.30	0.24	0.86	0.74	0.86	1.05	0.60	0.33	0.87	0.58	0.33	1.62	1.27	0.26	0.56	0.38	0.09
Toyota LA-4 BS2-1 trap	repeat	0.00	0.03	0.21	0.25	0.27	0.49	0.74	0.57	0.86	0.47										
	avg.	-0.01	0.04	0.20	0.28	0.26	0.68	0.74	0.72	0.96	0.54										
TAEB-84-0010	initial	0.06	0.40	0.67	0.60	1.22	1.03	1.79	1.33	2.18	3.49	0.46	0.67	1.04	0.83	2.61	1.32	0.81	1.00	1.35	0.19
Mercedes LA-4	repeat	0.01	0.11	0.41	0.59	1.15	1.20	1.60	1.30	3.49	3.38										
baseline	avg.	0.04	0.26	0.54	0.60	1.18	1.12	1.70	1.32	2.84	3.44										
TAEB-84-0020	initial	-0.03	0.39	4.37	1.59	2.69	3.03	12.47	2.54	14.50	12.21										
Mercedes LA-4	repeat	-0.03	0.34	5.60	2.60	4.34	3.31	11.97	3.86	13.63	12.09										
NGK-2 trap	avg.	-0.03	0.36	4.98	2.10	3.52	3.17	12.22	3.20	14.06	12.15										

[a] No repeat analysis
[b] Insufficient sample to perform repeat analysis
[c] Insufficient sample to perform analysis in TA 1535, TA 1537, TA 98

TABLE 5. RESULTS OF INDIVIDUAL AMES TESTS OF EXTRACTS, DOSE-RESPONSE RELATIONSHIP CALCULATED BY EPA-RTP NON-LINEAR MODEL

Sample Identification	Test	rev/µg																			
		TA 1535		TA 1537		A 1538		TA 98		TA 100		TA 97[a]		TA 102[a]		TA 194[a]		TA 98NR[a]		TA98/1,8-DNP6[a]	
		-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
TAEB-81-0781	initial	0.01	0.42	5.38	2.68	15.1	5.71	8.13	5.20	8.66	2.29	5.00	0.75	2.86	2.34	17.2	6.77	2.63	3.71	8.96	0.89
VWLA-4 baseline	repeat	0.07	0.51	0.84	4.45	6.77	4.22	17.6	4.86	6.30	4.85										
	avg.	0.04	0.47	3.11	3.57	11.0	4.97	12.8	5.03	7.48	3.57										
TAEB-82-0191[b,a] VW LA-4 JM-13 trap	initial	1.43	4.93	15.8	43.6	143	37.7	28.5	19.4	22.5	8.92										
TAEB-82-0101[b,a] VW LA-4 JM-13 trap	initial	0.76	2.81	8.24	15.9	27.9	36.0	14.9	7.66	13.9	6.64										
TAEB-82-0131[b,a] VW Regen. JM-13 trap	initial	--[c]	--[c]	--[c]	--[c]	--[c]	--[c]	4.54	24.7	4.47	9.36										
TAEB-82-0221	initial	0.00	0.35	1.55	0.33	1.70	0.96	1.79	1.47	1.58	1.17	1.09	1.06	2.54	1.82	3.41	2.48	0.30	5.28	0.95	0.46
Toyota LA-4 BS2-1	repeat	0.00	0.36	0.71	0.32	1.60	0.76	2.72	1.05	0.79	0.88										
trap	avg.	0.00	0.36	1.13	0.32	1.65	0.86	2.26	1.26	1.19	1.03										
TAEB-83-0161	initial	0.09	0.04	0.43	0.45	0.28	0.88	1.76	0.86	1.79	1.42	0.41	0.88	0.98	0.34	3.45	1.38	0.26	0.77	0.56	0.13
Toyota LA-4 BS2-1	repeat	0.01	0.04	0.44	0.24	0.76	1.22	1.62	1.80	1.36	0.96										
trap	avg.	0.05	0.04	0.44	0.35	0.52	1.05	1.69	1.33	1.58	1.19										
TAEB-84-0010	initial	0.00	0.45	12.9	1.17	4.48	2.00	5.66	3.35	4.91	7.07	2.18	1.93	20.6	1.34	7.19	2.60	1.26	2.92	9.25	0.16
Mercedes LA-4	repeat	0.00	0.47	1.73	0.84	6.52	1.98	5.98	2.12	11.0	8.08										
baseline	avg.	0.00	0.46	7.32	1.01	5.50	1.99	5.82	2.74	7.96	7.58										
TAEB-84-0020	initial	0.00	0.79	2.48	2.12	3.40	29.1	25.2	6.57	7.51	3.70										
Mercedes LA-4	repeat	0.00	0.93	--	12.6	4.93	28.4	41.8	10.2	1.13	2.72										
NGK-2 trap	avg.	0.00	0.86	2.48	7.36	4.17	28.8	33.5	8.39	60.3	3.21										

[a] No repeat analysis
[b] Insufficient sample to perform repeat analysis
[c] Insufficient sample to perform analysis in TA 1535, TA 1537, TA 98

III. ANALYTICAL PROCEDURES

The particulate filters provided by EPA were extracted by SwRI, and Ames bioassay of the organic extractables was performed by SFBR. Filter and extract processing was carried out under yellow lights. Each filter was extracted in a soxhlet apparatus with methylene chloride, at 4 cycles per hour for 8 hours, to provide a total of approximately 32 cycles per filter. The resulting organic extractables were filtered and concentrated under vacuum on a roto-evaporator. The extracts were further concentrated under a dry nitrogen purge and transferred to preweighed vials. The samples were weighed when dry, and percentages of extractables were calculated relative to particulate loading. The completed samples were then delivered to SFBR for Ames bioassay. The Ames bioassay procedure is included as an Appendix.

IV. RESULTS

Four of the extracts were produced by a Volkswagen Rabbit (VW), two were from a Toyota, and two from a Mercedes. As seen in Table 3, a relatively large fraction of the VW baseline filter sample was composed of organic extractables, compared to the trap and regeneration filters. Although the regeneration filter had relatively high particulate loading, less than one percent was organic soluble material. The two Toyota samples which were taken with a trap on the vehicle produced 30 and 26 percent extractables. The Mercedes baseline sample produced higher particulate levels than the Mercedes with trap, but a slightly lower fraction of extractables.

The results of the Ames bioassay by the non-linear model (in revertants per microgram of extract) generally exceeded the values determined by linear regression. The largest discrepancy between the two sets of data was with sample -0010, from the Mercedes baseline. Several of the non-linear model values were up to 20 times higher than linear values, as shown in Tables 1 and 2. For other samples, the non-linear values were up to five times higher than values determined by linear regression.

REFERENCES

1. Maron, D.M. and Ames, B.N., Revised Methods for the Salmonella Mutagenicity Test, Mutation Research, Vol. 113, (173-215) 1983.

APPENDIX

DETERMINATION OF THE IN VITRO GENOTOXICITY OF CHEMICALS BY THE SOUTHWEST FOUNDATION FOR RESEARCH AND EDUCATION

S. typhimurium Histidine Reversion Assay

Protocol 101

The Ames Salmonella typhimurium Histidine Reversion Assay

The Ames *Samonella typhimurium* histidine reversion assay is utilized on a regular basis at the Southwest Foundation for Research and Education. Mr. Arnaldo Noyola, who performs the bacterial mutagenesis procedure, received his training in conducting the assay in the laboratories of Dr. Thomas Matney at The University of Texas Health Science Center in Houston, TX, in 1977, and Dr. Vincent Simmon at SRI in Palo Alto, CA, in 1979.

The tester strains we are using have been obtained from the laboratories of Dr. Bruce Ames and Dr. Herbert Rosenkranz. These strains, as indicated in the Methods of Procedure section, are examined regularly using the diagnostic tests described by Ames et al. (1975), Levin et al. (1982), and Maron and Ames (1983) to insure the stability and integrity of the individual tester strains. The spontaneous mutation rate is regularly monitored.

For each sample, a minimum of five concentrations are tested in triplicate with each tester strain for evaluation of mutagenicity, both with and without *in vitro* metabolic activation. Tester strains TA 97, TA 98, TA 100, TA 102, and if requested, TA 1538, are routinely employed. Other tester strains that are available for testing include TA 1535, TA 1537, TA 98NR, TA 98/1,8-DNP_6, and TA 104. Also if requested (and at additional cost), a cytotoxicity assay will be performed at each concentration to determine bacterial concentration by a dilution procedure, so that revertants per surviving bacterium can be quantitated, in addition to revertants per plate.

Methods of Procedure

Preparation of Minimal Medium. Vogel-Bonner (VB) medium is prepared in three steps. First, a 50X concentrated VB salt solution is made by serial addition to 670 ml warm (45°C), glass distilled water of 10 g of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 100 g of citric acid monohydrate, 500 g potassium phosphate (dibasic), and 175 g sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$). Special attention is given to the requirement that each chemical is added and dissolved successively. The final 50X salt concentrate is then aliquoted into 40 and 80 ml portions into storage bottles are are loosely capped and autoclaved for 15 min at 250°F at 15 p.s.i. The second step in medium formulation involves preparation of a 40% glucose solution. Four hundred grams of dextrose is dissolved in warm (45°C), glass distilled water and the volume is adjusted to 1 liter. One-hundred milliliter aliquots are transferred into storage bottles that are loosely capped and autoclaved as above. In the final step of medium formulation, 30 g of Difco Agar is dissolved in 1800 ml glass distilled water and autoclaved. The sterile agar is then allowed to cool to 70°C in a constant temperature bath, and 40 ml of the 50X salt solution and 100 ml of the 40% glucose solution are added. The final medium is thoroughly mixed and 25 ml portions are poured into sterile 100 x 15 mm petri dishes. The plates are allowed to gel at room temperature on a level surface prior to storage, after inversion, at 4°C.

Preparation of Top Agar

The procedure used for preparation of the top agar is similar to that described above. The top agar is prepared by adding 6 g of Bactoagar and 5 g

of NaCl to 1 liter of distilled water in a 2-liter flask. The final agar concentration is 0.6% and the final NaCl concentration is 0.5%. The contents of the flask are brought to boiling on a heated magnetic stirrer. Aliquots of 100 ml are then transferred into milk dilution bottles and loosely capped; they are then autoclaved for 15 min at 250°F and 15 psi. The caps are tightened prior to removal of the bottles from the autoclave. The bottles are allowed to stand at room temperature until the agar gels, and they are stored at room temperature prior to usage.

For each experiment, bottles containing an appropriate amount of top agar are reheated in the autoclave for 6 min at the same temperature and pressure as described above. The bottles are then placed in a 45°C oven and are allowed to temperature equilibrate for approximately 2 hr. The melted top agar is supplemented with 10 ml of a sterile 0.5 mM L-histidine HCl-0.5 mM biotin solution for each 100 ml of top agar, and 2-ml aliquots of the supplemented top agar, are distributed into sterile capped 13 x 100 mm test tubes.

Preparation of Chemicals for the Plate Incorporation Assay

All chemicals, whether known or unknown, are carefully weighed in a vented glove box housing a five-place Mettler balance. The chemicals are weighed in individual sterile 13 x 100 mm capped test tubes. The chemicals are transferred from the glove box into the vented hood and placed into solution using the proper amount of a sterile solvent (usually DMSO). The stock chemicals are further diluted into sterile capped tubes containing the appropriate volumes of sterile solvent.

The dilutions are arranged so that the amount of chemical to be added per plate will be contained in 0.1 ml. The compounds are generally tested for mutagenicity at levels between 0.01 and 1000 µg per plate. Sufficient material is placed in solution so that triplicate plates can be run at each concentration, with and without S9 present for metabolic activation.

Preparation of the S9 Mix (metabolic activation system)

Male Sprague-Dawley rats, weighing approximately 200 g, are given an i.p. injection of Aroclor 1254 (a polychlorinated biphenyl mixture), diluted in corn oil to a concentration of 200 mg/ml, at a dosage of 500 mg/kg of rat weight 5 days prior to sacrifice. Twelve hours prior to sacrifice, the food is removed and, on the fifth day, the rats are stunned by a blow to the head and decapitated. Using sterile techniques, the liver is removed and weighed in a preweighed sterile beaker containing ice cold 0.15 M KCl. The livers are subsequently washed with one volume of cold, sterile 0.15 M KCl per weight of wet liver, then placed in three volumes of cold sterile 0.15 M KCl per weight of liver. The liver is minced with sterile scissors, transferred into a sterile tissue homogenizer (Tissuemizer), and homogenized. The homogenate is then centrifuged in sterile capped tubes at 9000 g (8700 rpm) for 10 min in a Sorval RC2-B centrifuge. The supernatant (S9) fraction is aliquoted in 3 or 8.5 ml volumes into sterile capped polypropylene tubes that are immediately frozen in dry ice. The tubes are stored until needed in a -80°C Revco freezer.

Preparation of Cofactors

The cofactor solution consists of D-glucose-6-phosphate, NADP monosodium salt, magnesium chloride, potassium chloride, monobasic and dibasic sodium phosphate. For each 100 ml of cofactor solution, 0.157 g D-glucose-6-PO_4, 0.350 g NADP, 0.180 g $MgCl_2$, 0.273 g KCl, 1.278 g dibasic sodium phosphate, and 0.278 g monobasic sodium phosphate are added to glass distilled water. When all the chemicals are in solution, the volume is adjusted to 100 ml and the solution is sterile-filtered with a disposable 100 ml Nalgene filter unit. The sterile cofactor solution is dispensed into sterile screw-capped tissue culture tubes in 9-ml aliquots, quickly frozen, and stored at -20°C. Upon determination of the amount of S9 mix needed for a given assay, appropriate amounts of S9 homogenate and cofactor solutions are thawed and mixed at a ratio at 1 ml S9 homogenate to 9 ml of cofactor solution.

Preparation of the Bacterial Culture

The procedures described herein apply to all S. typhimurium tester strains. The bacterial tester strains are removed from the Revco freezer and inoculated into separate 16 x 180 mm Kim-capped test tubes, each containing 5 ml of Oxoid nutrient broth. Inoculation is performed by transferring 20 µl of a thawed bacterial culture into the Oxoid nutrient broth. The Oxoid broth is prepared by dissolving 25 g of Oxoid nutrient broth No. 2 in 1 liter of distilled water and autoclaving.

The tester strains are inoculated into the Oxoid nutrient broth at 4:30 p.m. and incubated overnight at 37°C, with agitation, in an incubator. At

8:30 a.m. the following morning, when the assay is to be conducted, the bacterial strains are removed from the incubator and placed on ice while the other necessary preparations for the assay are completed. Occasionally, an aliquot is removed to determine the bacterial concentration spectrophotometrically at 625 nm.

The Plate Incorporation Assay

While the tester strains are on ice, the top agar is melted, supplemented with histidine and biotin, and then distributed in 2 ml aliquots into 13 x 100 mm test tubes which are placed in a multiblock heater maintained at a temperature of 45°C. The activated S9 mix is freshly prepared as described above and chilled on ice. The chemicals are weighed out, placed in solution, and diluted to different concentrations as previously described. The minimal medium plates are properly labeled in triplicate and brought into the hood in groups of ten. The refrigerated tester strains and the S9 mix which are kept chilled in ice are also brought inside the vented hood. The usual procedure is to first inoculate each 13 x 100 mm text tube containing molten top agar with 0.1 ml of organism, followed by 0.1 ml of chemical and, if required, 0.5 ml of activated S9 mix. The test tube is then removed from the multiblock heater and vortexed for 2 sec; the tube contents are then poured into the appropriate prelabeled plate, which is gently tilted to spread the top agar over the entire surface of the minimal medium plate. The plates are allowed to stand in the hood at room temperature for gelling while the other plates are poured in the same manner. After all the plates have been poured and the top agar overlay has been allowed to gel for a minimum of 30 min, the plates

are inverted in groups of ten, removed from the vented hood, and placed in a vented Forma reach-in incubator set at 37°C for 48 hr incubation.

After 48 hr, the plates are removed from the incubator and the revertants on each plate are counted using an electronic counter (NBS Model C111). In our laboratory, the spontaneous mutation rates of solvent controls in all tester strains is similar to those reported by Maron and Ames (1983). With the activated S9 system present, 2-aminofluorene is regularly used as a positive control. NaN_3 (TA 100, TA 1535), methyl methane sulfonate (TA 102), and 2-nitrofluorene (TA 98, TA 1538), crotonaldehyde (TA 104), ICR-191 (TA 97), and 9-aminoacridine (TA 1537) are positive controls for testing without added S9.

Testing of Bacterial Strain Sensitivity for Isolation of Desirable Tester Strain Stock

Diagostic tests to confirm the stability and integrity of our tester strains are performed regularly. For this purpose, samples of the tester strains are inoculated into 5 ml of Oxoid nutrient broth and allowed to grow for approximately 16 hr in a 37°C incubator. The following day, the bacteria are streaked on sterile 100 mm petri plates with 25 ml of nutrient agar and incubated for 24 hr at 37°C to recover single cell isolates of each of the tester strains. After 24 hr, three single cell isolates of each tester strain are inoculated into three separate 18 x 150 mm test tubes containing 5 ml of Oxoid nutrient broth then incubated overnight. Three samples of each tester strain are then available for sensitivity testing.

Nutrient agar plates are used to assay for crystal violet, ampicillin, and UV sensitivity. An aliquot (0.1 ml) of each culture is added to 2.0 ml of nutrient top agar, mixed, and poured onto a prelabeled nutrient agar plate. With sterile forceps, a crystal violet disc (0.25 inch with 10 µl of a 1 mg/ml crystal violet solution) is placed in the center of each plate. The plates are then incubated overnight, and the zone of inhibition around the disc is determined. A strain is acceptable (i.e., has a good rfa) if the zone of inhibition is 14 mm or greater.

To determine ampicillin resistance, 0.1 ml of a sterile solution of 8 mg/ml ampicillin in 0.02 N NaOH is spread to dryness on two nutrient agar plates. Two plates without ampicillin serve as the controls for ampicillin resistance and UV sensitivity test as described below. Each of the four plates is divided into eight sections, and three samples of each of the five strains are streaked on ampicillin plus and ampicillin minus sections. The plates are incubated overnight at 37°C to determine the ability to grow in the presence of ampicillin.

To determine the presence of the tetracycline resistance gene in TA 102, plates containing ampicillin and 2 µg/ml of an 8 mg/ml tetracycline solution in 0.02 N HCl are used. The bacterial cultures are streaked in the same manner as the ampicillin test plates and an R-factor strain (i.e., TA 100) is used as a control. The plates are placed in an incubator overnight at 37°C, and the tetracycline resistance gene is confirmed by the growth capability. TA 100, which lacks the gene, is sensitive to tetracycline, while TA 102 is resistant.

To determine UV sensitivity, nutrient agar plates sectioned into eight areas and all 15 samples are streaked across the sectioned plates in parallel strips. A piece of cardboard is placed over the uncovered plate so that half of each bacterial streak is covered. A UV lamp, warmed and functioning for a minimum of 15 min, is placed approximately 33 cm from the top of the plates and the covers of the plates are removed prior to exposure. Non-R-factor strains (i.e., TA 1538) are irradiated for 6 sec and R-factor strains (i.e., TA 98) are irradiated for 8 sec. TA 102 is used on the same plates as a control for the UV exposure. After exposure, the plates are incubated overnight at 37°C, and examined for evidence of sensitivity to UV by the presence or absence of growth.

To test for the other diagnostic requirements, such as histidine and biotin, or spontaneous mutation rates, minimal medium agar plates are employed. For spontaneous reversion rates, each sample is assayed in triplicate; therefore, 45 minimal medium plates plus 45 test tubes (13 x 100) with 2 ml of top agar (supplemented with biotin-histidine) are used. To 2 ml of top agar, 0.1 ml of the bacterial culture is added, vortexed, and poured onto a properly labeled petri dish containing minimal medium. The plates are incubated at 37°C for 48 hr, after which time the revertant colonies are counted. Acceptable levels of spontaneous reversion in our laboratory are TA 1538: 15-35; TA 97: 90-180; TA 98: 20-50; TA 100: 100-200; TA 102: 240-320, and TA 104: 275-425. When the number of spontaneous revertants per plate fails to fall within the acceptable range, new stocks are prepared by selection of clones containing the desired activity. The new stocks are aliquoted and stored frozen at -80°C.

To test for histidine and biotin requirement, sterile stock solutions of histidine (1 g/100 ml glass distilled water) and biotin (10 mg/100 ml water) are used. To three minimal medium agar plates, 0.1 ml of the histidine solution is spread throughout the surface of the plates with an L-shaped glass rod until they are dry. To a second set of plates, 0.1 ml of the biotin solution is similarly added. To the third set of plates, 0.1 ml of the histidine solution is spread until dry, followed by 0.1 ml of the biotin solution that is also spread until dry. The plates are divided into eight sections, streaked with the cultures as described above, and incubated overnight at 37°C to determine the presence of normal growth.

For each of the 15 isolates tested for crystal violet, UV, ampicillin, tetracycline, histidine and biotin sensitivities, and spontaneous reversion rate, a data chart is constructed. The zones of inhibition around the crystal violet disk are measured and the values are entered into the data chart. All tester strains should be sensitive to UV, except TA 102, and they should be able to grow in the combination of histidine and biotin. TA 1538 should be sensitive to ampicillin; TA 98 and TA 100 should be resistant to ampicillin. TA 102 is the only strain which is resistant to tetracycline. From the information in the chart, the single cell isolates which are most representative of published and our own historically acceptable values are used to prepare stock cultures for storage. Isolates are inoculated into culture tubes with 5 ml nutrient broth, then incubated overnight at 37°C. After 16 hr growth, the tester strain cultures are diluted with 0.09 ml of DMSO for each ml of culture, quickly frozen in dry ice, and stored in a Revco freezer at -80°C.

REFERENCES

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